REMARKS

Status of the Claims.

Claims 1-13 are pending with entry of this amendment, claims 14-81 being cancelled and no claims being added herein. Claims 1, 3, 4, and 13 are amended herein. These amendments introduce no new matter. Support is replete throughout the specification, for example, in the claims as filed, at page 66, lines 3-6, at page 67, lines 1-5, and so forth.

Election/Restriction.

Pursuant to a restriction requirement made final, Applicants cancel claims 16-81 with entry of this amendment. Please note, however, that Applicants reserve the right to file subsequent applications claiming the canceled subject matter and the claim cancellations should not be construed as abandonment or agreement with the Examiner's position in the Office Action.

35 U.S.C. §112, Second Paragraph.

Claim 4 was rejected under 35 U.S.C. §112, second paragraph, as allegedly indefinite because it was allegedly unclear if the phospholipid is a complex with the claimed fatty acids or the claimed fatty acids are additional agents in the formulation. Applicants traverse.

The Examiner is reminded that a claim is definite if "... read in light of the specification [it] reasonably apprise[s] those skilled in the art both of the utilization and scope of the invention, and if the language is as precise as the subject matter permits. *Hybritech Inc. v Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1385, 231 USPQ 81 (Fed. Cir. 1986) *cert. denied* 480 U.S. 947 (1987).35 U.S.C. §112, first paragraph.

In the instant case, claim 1 (incorporating language formerly present in claim 4) recites

> . . . wherein-said phospholipid is a synthetic phospholipid selected from the group consisting of phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, and phosphatidyl inositol**, having fatty acids in the *sn-1* and *sn-2* positions that are the same and that range in length from 3 to 24 carbons**.

The "class" of phospholipids (*e.g.*, phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, phosphatidyl inositol, *etc.*) is determined by the moiety in the *sn-3* position of the phospholipids. Different fatty acids can exist in the sn-1 and sn-2 positions of the phospholipids

thereby changing the species, but not the genus. Thus, for example, a phosphatidyl choline can have a propionoyl, a butanoyl, a pentanoyl, and so forth in the *sn-1* and/or *sn-2* position.

One of ordinary skill in the art, reading claim 1, would readily recognize that the recited fatty acids are moieties occupying certain positions in the lipid thereby forming a particular species of phospholipids. The claim language is consistent with conventional nomenclature.

The claims thus reasonably apprise those skilled in the art both of the utilization and scope of the invention, and are as precise as the subject matter permits. Accordingly, the rejection under 35 U.S.C. §112, second paragraph should be withdrawn.

35 U.S.C. §102.

Claims -3, 6-8, 10-12, 14 and 15 were rejected under 35 U.S.C. §102(b) as allegedly anticipated by Hsia *et al.* (U.S. Patent 5,231,090). The Examiner alleged that Hsia *et al.* teaches that phospholipids administered intravenously and topically result in the resolution of atherosclerotic lesions. The Examiner further argued that upregulation of an MKP-1 gene is inherent in the teachings set for the in Hsia *et al.*. Applicants traverse by argument and amendment.

Claim 1, as amended herein recites:

> Claim 1 (Original): A method of **raising HDL level and increasing HDL anti-inflammatory activity** in a mammal, said method comprising administering to said mammal a phospholipid in an amount sufficient to increase HDL level in said mammal, wherein-said phospholipid is a **synthetic** phospholipid **selected from the group consisting of phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, and phosphatidyl inositol, having fatty acids in the sn-1 and sn-2 positions that are the same and that range in length from 3 to 24 carbons.**

Hsia *et al.* fails to identify or in any way disclose at least two features of the presently pending claims. Thus, for example Hsia *et al.* fails to disclose:

1) **Synthetic phospholipids** that that are phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, or phosphatidyl inositol, **having fatty acids in the *sn-1* and *sn-2* positions that are the same** and that range in length from 3 to 24 carbons;

and

2) That these phospholipids can raise HDL level and **increase HDL anti-inflammatory activity**.

To the contrary, Hsia *et al.* simply discloses the use of phospholipids for increasing HDL cholesterol. No mention whatsoever is made regarding HDL protein levels or HDL anti-inflammatory activity.

Moreover, the claimed method is not inherent in the Hsia *et al.* disclosure. Hsia *et al.* expressly teach that lecithin works in their claimed method and is preferred:

> Phospholipid-containing compositions suitable for use in the present method comprise at least one phospholipid in a concentration of between, for example, 5% and 50% (w/v). Any natural or synthetic phospholipid, **lecithin (phosphatidylcholine)**, phosphatidylethanolamine and phosphatidylserine, having an affinity for cholesterol can be used in the composition, however, **lecithin is preferred**. [emphasis added] (col. 2, lines 56-63).

It is noted that Hsia *et al.* used **naturally occuring** lecithin extracted from egg yolk (*see,* col., 3, lines 38-41), not a synthetic phospholipid as recited in the pending claims.

Naturally occurring phospholipids (*e.g.*, egg yolk lecithin) typically **do not** have the same fatty acid in the *sn-1* and *sn-2* position. Moreover, it is noted that such "naturally occurring" lecithin **does not work in the presently claimed methods.**

A paper by Navab *et al.* (Navab *et al.* (2003) *Circulation,* 108: 1735-1739), submitted herewith as Exhibit A, provides a comparison of egg yolk lecithin, soy lecithin, and DMPC in their ability to increase HDL function. As stated therein:

> In other experiments, apoE-null mice were given drinking water alone, drinking water containing DMPC, or soy lecithin or egg lecithin, all at 1.0 mg/mL. After 8 weeks, there were no significant differences in plasma triglyceride levels for the mice in the different treatment groups (water, 142±16 mg/dL; egg lecithin, 138±18 g/dL; soy lecithin, 161.6±8.5 mg/dL; and DMPC, 158.4±6.3 mg/dL). Total cholesterol was also not different (water, 569±97 mg/dL; egg lecithin, 549±132 mg/dL; soy lecithin, 576±86 mg/dL; and DMPC, 587±112 mg/dL). Total plasma phospholipid levels were not significantly different among the groups (water group, 318±28 mg/dL; soy lecithin, 332±17 mg/dL; egg lecithin, 327±23 mg/dL; and DMPC, 341±27 mg/dL). **HDL phospholipid levels were 85.2±7.3, 88.1±2.9,**

> **84.2±2.9, and 200.5±11.3 mg/dL in the groups on water, egg lecithin, soy lecithin, and DMPC, respectively, indicating a 2.3-fold increase (*P*±0.003) in plasma HDL phospholipids in the mice receiving DMPC**. DMPC was not found in the plasma of any mice except those given DMPC. Plasma DMPC concentration in the group that received DMPC in their drinking water was 84±74 μg/dL, or 0.02% of the total plasma phospholipid concentration. Plasma free fatty acid concentrations after an overnight fast were not significantly different between the groups. [emphasis added] (col. 2, page 1736).
>
> * * *
>
> As shown in Figure 2, when DMPC or soy or egg-yolk lecithin at 1.0 mg/mL was added to the drinking water of 4-week-old apoE-null mice for 8 weeks, **HDL function was dramatically improved in the mice receiving DMPC but not in those receiving lecithin.**
>
> Figure 3 demonstrates that there was **a significant reduction in aortic sinus lesions in the mice receiving DMPC but not in the mice receiving either egg-yolk or soy lecithin.** [emphasis added] (page 1737, col. 1)

The presently claimed method is simply not inherent in Hsia *et al.* as the Navab *et al.* reference (Exhibit A) demonstrates that egg-yolk lecithin **does not** function to produce the result recited claimed method.

Because Hsia *et al.* fails to disclose synthetic phospholipids having the same fatty acid at positions *sn-1* and *sn-2,* because Hsia *et al.* offers no teaching that such phospholipids can increase HDL level and anti-inflammatory activity, and because Hsia *et al.* illustrates methods utilizing phospholipids that **do not** function in the presently claimed methods, this reference neither expressly nor inherently anticipates the presently pending claims. Accordingly, this rejection under 35 U.S.C. §102(b) should be withdrawn.

35 U.S.C. §103(a).

Claim 9 were rejected under 35 U.S.C. §103(a) as allegedly obvious in light of Hsia *et al.* (U.S. Patent 5,231,090) in view of Bertelli (U.S. Patent 4,684,520). The Examiner states that Hsia *et al.* fails to teach or suggest oral administration and relies on Bertelli as allegedly teaching oral

administration of phospholipids. Claim 5 was rejected under 35 U.S.C. §103(a) as allegedly obvious in light of Bertelli. The Examiner stated that Bertelli teaches that 1,2-dimyristoyl-sn-glycero-3-phosphocholine is useful in the treatment of atherosclerosis. Applicants traverse.

A *prima facie* case of obviousness requires that the combination of the cited art, taken with general knowledge in the field, must provide all of the elements of the claimed invention. When a rejection depends on a combination of prior art references, there must be some teaching, suggestion, or motivation to combine the references. *In re Geiger*, 815 2 USPQ2d 1276, 1278 (Fed. Cir. 1987). Moreover, to support an obviousness rejection, the cited references must additionally provide a reasonable expectation of success. *In re Vaeck*, 20 USPQ2d 1438 (Fed. Cir. 1991), *citing In re Dow Chemical Co.*, 5 USPQ2d 1529, 1531 (Fed. Cir. 1988).

Moreover it is well recognized that an obviousness rejection requires **a particularized teaching or suggestion to combine elements** to practice the claimed methods or to make the claimed compositions or kits. As stated by the Court of Appeals for the Federal Circuit:

> Our case law makes clear that the best defense against hindsight-based obviousness analysis is the rigorous application of the requirement for a showing of a teaching or motivation to combine the prior art references. See Dembiczak, 175 F.3d at 999, 50 USPQ2d at 1617. **"Combining prior art references without evidence of such a suggestion, teaching, or motivation simply takes the inventor's disclosure as a blueprint for piecing together the prior art to defeat patentability--the essence of hindsight." Id.** [emphasis added] *Ecolochem, Inc. v Southern-California Edison Company,* __ USPQ2d ___ (Fed. Cir. 2000)
>
> * * *
>
> The mere fact that the prior art may be modified in the manner suggested by the Examiner **does not** make the modification obvious unless the prior art suggested the desirability of the modification. [emphasis added] *In re Fritch*, 23 USPQ 2d 1780, 1783-1784 (Fed. Cir. 1992)

In the instant case, as explained above, Hsia *et al.* offers no teaching or disclosure that **synthetic** phospholipids comprising the same fatty acid in the *sn-1* and *sn-2* positions raise HDL level and increase HDL anti-inflammatory activity. Moreover, Hsia *et al.* teaches away from the presently claimed invention by teaching the preferred use of egg lecithin, a phospholipid that **does not** function in the presently claimed methods.

This defect is not remedied by either Aviram *et al.* or Bertelli. Aviram *et al.* simply discloses the inhibition of lipoprotein oxidation using hydroxylated derivatives of known cholesterol lowering agents (*see, e.g.,* col. 1, lines 8-11). In particular, Aviram *et al.* teaches the use of hydroxylated statins or hydroxylated fibrates to inhibit lipoprotein oxidation. There is no teaching or suggestion of the use of synthetic phospholipids to increase HDL level or HDL anti-inflammatory activity. Indeed, there is no teaching or suggestion of the use of a phospholipid as in this context.

Similarly, Bertelli discloses the use of ubiquinone (*e.g.,* coenzyme Q_{10} and mixtures of **naturally occurring** phospholipids) to treat a variety of conditions including ". . . atherosclerosis, fatigability, loss of memory, neuroendocrine diseases, and generally in the conditions deriving from cerebral and tissular postanoxy." (*see, e.g.,* col. 1, lines 34-37). There is no teaching or suggestion of the use of synthetic phospholipids to increase HDL level or HDL anti-inflammatory activity.

To the contrary, Bertelli *et al.* expressly leads one of skill away from the presently clamed invention. The pending claims are directed to methods utilizing

> . . . a **synthetic phospholipid** selected from the group consisting of phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, and phosphatidyl inositol, **having fatty acids in the *sn-1* and *sn-2* positions that are the same and that range in length from 3 to 24 carbons**. [emphasis added] (see claim 1)

In contrast, Bertelli *et al.* expressly states:

> In fact, the **combination of Coenzyme Q_{10} and a mixture of phospholipids** (phosphatidylcholine, phosphatidilethanolamine, sphyngomieline, phosphatidylserine, phosphatidylinositol) or **soy lecithin** (Coenzyme Q_{10} 50 mg/kg o + phospholipid mixture 500 g/kg or **soy lecithin** 500 mg/kg, by oral route) inhbit the formation of atherosclerotic lesions in a substantially more effective way than the administration of Coenzyme Q_{10} alone or phospholipids or soy lecithin alone. [emphasis added] (col. 5, lines 44-52)
>
> * * *
>
> It was noticed that while the control rats as well as **the rats treated with Coenzyme Q_{10} alone or phospholipids or soy lecithin alone had serious atherosclerotic lesions** at the aortic and myocadial level, the rats treated with

> Coenzyme Q10 and phospholipids or lecithin showed no or negligible atherosclerotic lesions. [emphasis added] (col. 5, line 63, to col. 6, line 3)

Bertelli thus leads one of skill away from the use of phospholipids per se to the use of Coenzyme Q_{10} as an active ingredient. Moreover, Bertelli leads one of skill to the use of naturally occurring phospholipids (soy lecithin) **in combination** with Coenzyme Q_{10}.

It is also noted that Bertelli offers no teaching or suggestion whatsoever regarding HDL level or HDL anti-inflammatory activity.

The combination of the cited art thus simply fails to teach or suggest the presently claimed methods. Accordingly the rejections under 35 U.S.C. §103(a) should be withdrawn.

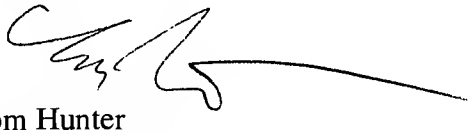
With respect to the requirement under 37 C.F.R. §1.56, Applicants believe that all pending claims were commonly owned at the time of invention and to the present date.

In view of the foregoing, Applicants believe all claims now pending in this application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested. Should the Examiner seek to maintain the rejections, Applicants request a telephone interview with the Examiner and the Examiner's supervisor.

If a telephone conference would expedite prosecution of this application, the Examiner is invited to telephone the undersigned at (510) 769-3513.

QUINE INTELLECTUAL PROPERTY LAW
GROUP, P.C.
P.O. BOX 458
Alameda, CA 94501
Tel: 510 337-7871
Fax: 510 337-7877

Respectfully submitted,



Tom Hunter
Reg. No: 38,498

Oral Synthetic Phospholipid (DMPC) Raises High-Density Lipoprotein Cholesterol Levels, Improves High-Density Lipoprotein Function, and Markedly Reduces Atherosclerosis in Apolipoprotein E–Null Mice

Mohamad Navab, PhD; Susan Hama, BSc; Greg Hough, MS; Alan M. Fogelman, MD

Background—Lecithin has been widely sold as a dietary supplement. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) is a phospholipid that does not exist in nature and has been used in vitro to study lipid binding. We tested DMPC in vivo in apolipoprotein (apo) E–null mice.

Methods and Results—DMPC or soy or egg lecithin at 1.0 mg/mL was added to the drinking water of 4-week-old apoE-null female mice. Eight weeks later, HDL cholesterol levels and apoA-I levels were markedly increased in the mice that received DMPC. HDL function was also dramatically improved in the mice receiving DMPC, and there was a significant reduction in aortic lesions ($P=0.021$) in the DMPC mice but not in those receiving lecithin. Adding 1.0 mg/mL of DMPC to the drinking water of 10-month-old apoE-null female mice for 5 weeks caused regression of aortic sinus lesions ($P=0.003$). Adding 1.0 mg/mL DMPC to the drinking water of 6-month-old apoE-null male mice for 8 weeks significantly reduced aortic sinus lesion area ($P=0.0031$) and en face whole aorta lesion area ($P=0.001$), whereas adding the same concentrations of soy or egg lecithin did not significantly alter lesion area. Jejunal apoA-I synthesis and plasma apoA-I levels were increased 2- to 3-fold in mice receiving DMPC but not soy or egg lecithin.

Conclusions—DMPC (but not lecithin) raises HDL cholesterol and apoA-I, improves HDL function, and prevents lesions or causes their regression in apoE-null mice. (***Circulation.* 2003;108:1735-1739.**)

Key Words: atherosclerosis ■ lipids ■ lipoproteins

There are many in vitro studies in the literature reporting on the characteristics of the association of apolipoprotein A-I (apoA-I) with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), such as that reported by Huang et al.[1] On searching the literature, we were unable to find any study in which DMPC was fed to animals or humans. Although DMPC has not been studied as an oral agent, lecithin has been widely sold as a dietary supplement for the prevention of a number of diseases, including atherosclerosis, for many years. The lecithin that has been fed to animals and humans has been derived primarily from soybeans or egg yolk.[2] Studies in animals and humans have provided conflicting results.[3–11] In 1982, the Food and Drug Administration asked the Federation of American Societies for Experimental Biology to review the status of development and utilization of high levels of lecithin, phosphatidylcholine, and choline as dietary supplements and the possible benefits or hazards from their consumption. The review concluded that in general, humans tolerate ≈25 g/d of commercial lecithin without side effects. It was noted that 60 to 80 g/d of phosphatidylcholine (85% purity) was given to some patients without difficulty, and none had side effects up to 40 g/d.[12]

Another review of 24 studies on the effect of supplementary lecithin with intakes ranging from 1 to 54 g/d revealed that most studies lacked an appropriate control group, had a small sample size, or had changes in intake of foods because of increased energy intake from lecithin.[13] The authors concluded that there was no evidence for a specific effect of lecithin on serum cholesterol independent of its linoleic acid content or secondary changes in food intake.[13]

In vitro, phosphatidylcholine was shown to have no effect on apoB secretion but significantly increased the synthesis and basolateral secretion of apoA-I in a newborn swine intestinal epithelial cell line.[14] In vivo, feeding soybean phosphatidylcholine to newborn swine approximately doubled the synthesis of apoA-I in the jejunum without altering apoB or apoA-IV synthesis.[15] On searching the literature, we also found no studies of phospholipids given orally to more modern animal models of atherosclerosis, such as apoE-null mice. We report here that feeding DMPC to apoE-null mice has a profound effect on HDL cholesterol, HDL function, and atherosclerosis.

Received October 23, 2002; de novo received April 11, 2003; revision received June 13, 2003; accepted June 18, 2003.

From the Department of Medicine, David Geffen School of Medicine at the University of California Los Angeles, Los Angeles, Calif.

Authors M. Navab, S. Hama, and A.M. Fogelman are principals in BruinPharma, a start-up biotech company.

Correspondence to Mohamad Navab, PhD, Room 47-123 CHS, Division of Cardiology, Department of Medicine, David Geffen School of Medicine at UCLA, 10833 Le Conte Ave, Los Angeles, CA 90095-1679. E-mail mnavab@mednet.ucla.edu

Circulation is available at http://www.circulationaha.org

DOI: 10.1161/01.CIR.0000089375.60050.35

Methods

Materials

DMPC (catalogue No 850345), egg-yolk lecithin (catalogue No 830051), soy lecithin (catalogue No 830054), and 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphorylcholine (PAPC) (catalogue No 850459) were purchased from Avanti Polar Lipids. 13(*S*)-HPODE was purchased from Biomol. Rabbit polyclonal antimouse apoA-I antibody, affinity-purified (catalogue No K23001R), was purchased from Biodesign International. All other materials were from previously cited sources.[16]

Mice

Male and female apoE-null mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and were maintained on a chow diet (Ralston Purina). The lipids were weighed and added to the drinking water and mixed (vortexed for 2 minutes) to make a uniform suspension. The DMPC, obtained in the form of a fine powder, readily resulted in a uniform suspension when mixed in water. Egg lecithin and soy lecithin also formed uniform suspensions after vortexing. The suspension was added to 50-mL graduated polypropylene tubes fitted with standard rubber stoppers and stainless steel tubes of a type used routinely in mouse water bottles. The tubes were observed daily, and the lipids were found by inspection to remain in suspension. The contents of the tubes were changed every other day, and 98.2±11% of the added phospholipid was determined by chemical analysis to still be in suspension at the time of the tube change. The mice drank 2 to 2.5 mL/d per mouse, and there was no significant difference in the amount of suspension consumed between the groups.

Intestinal apoA-I synthesis was determined as described previously.[15] Briefly, mice (4 animals per group) that had been fasted overnight were anesthetized with isoflurane by use of an anesthesia machine in accordance with the policies and with the approval of the UCLA Animal Research Committee. The abdomen was opened and the intestine exposed. The jejunum was isolated distal to the duodenum and cut laterally with a small scissors, a ball-end needle was inserted, and the jejunum/duodenum was ligated. The proximal end of the ileum was clamped. Radiolabeling was performed by instilling 0.25 mCi (0.25 mL) of L-[4,5-^{3}H]leucine (>120 Ci/mmol, Amersham) into the segment for 9 minutes. Body temperature was maintained by a recirculating water table. The animal was then killed by isoflurane overdose, the jejunum segment was removed and opened, and the mucosa was removed, homogenized, and subjected to immunoprecipitation. After SDS-PAGE, the newly synthesized apoA-I was determined after subtracting nonspecific counts determined from incubations with nonimmune antibody, dividing by TCA-precipitable counts, and normalizing the control to 1.0.

Lipoproteins, Cocultures, Monocyte Chemotaxis, and Lesion Scoring

Lipoproteins, cocultures, and monocytes were prepared, and monocyte chemotaxis assays were performed as described previously.[16,17] Plasma samples were fractionated by a gel-permeation fast protein liquid chromatography (FPLC) system consisting of dual Pharmacia Superose 6 columns in series. Plasma (≤0.45 mL) was eluted with an isocratic buffer containing 154 mmol/L NaCl, 1 mmol/L EDTA, and 0.02% sodium azide, pH 8.2, at a flow rate of 0.5 mL/min, pumped by a nonmetallic Beckman high-performance liquid chromatography pump. Forty-two 1-mL fractions were collected. Fractions containing lipoproteins isolated in the absence of EDTA contained 20 μmol/L BHT. Aortic lesions were scored as described previously.[18,19]

Other Procedures

Plasma phospholipid levels were determined with a kit from Wako Chemicals (No. 990-54009E) and by mass spectrometry/mass spectrometry according to the protocols described previously.[20,21] Paraoxonase activity was measured as described previously.[22] Plasma free fatty acid concentrations were measured with a commercial kit (Wako) on the basis of a previously published method.[23] Lipoprotein cholesterol concentrations were determined with a Cholesterol-20 kit (Sigma). Plasma apoA-I levels were determined by a competitive binding ELISA and by Western blot analysis. ELISA plates were obtained from Corning Costar, coated with murine apoA-I (A23100M from Biodesign International), and blocked with gelatin (G-1890 Sigma). A standard curve was constructed with murine apoA-I (A23100M from Biodesign International), and the assay included internal standards. Primary antibody was affinity-purified rabbit anti-mouse apoA-I (K23001R from Biodesign International), and the secondary antibody-conjugate was horseradish peroxidase–labeled anti-rabbit IgG F(ab′)2 (Jackson Immunoresearch). TMB peroxidase substrate was from Kirkegaard and Perry Laboratories. The reaction was terminated with sulfuric acid, and the absorbance was read at 450 nm. In Western analyses, murine apoA-I plasma (0.5 μL) was subjected to SDS-PAGE (4% to 20% Tris glycine from Novex) and Western transferred (semidry onto nitrocellulose from Amersham). The blots were treated sequentially with rabbit anti-mouse apoA-I (Biodesign International K23500R), horseradish peroxidase–labeled anti-rabbit IgG F(ab′)2 (Jackson), and Amersham ECL reagent and exposed to film (Amersham). The films were scanned with a Molecular Dynamics densitometer. Murine apoA-I (0.075 μg) was included as an internal standard. Statistical significance was determined using model I ANOVA, and significance was defined as a value of $P<0.05$.

Results

Twenty-four hours after DMPC (but not lecithin) had been added to the drinking water, cholesterol-containing particles with paraoxonase 1 activity appeared in the region to the right of HDL in the FPLC chromatogram (data not shown). After 24 hours, the lipids were removed from the drinking water, and the mice were bled 24 hours later, ie, 48 hours after the start of the experiment. After this second 24-hour "chase" period, the cholesterol peak containing paraoxonase 1 activity, which at 24 hours was found to the right of HDL in the FPLC chromatogram, moved into the mature HDL region, and the HDL shifted from proinflammatory to antiinflammatory in the coculture assay (data not shown).

In other experiments, apoE-null mice were given drinking water alone, drinking water containing DMPC, or soy lecithin or egg lecithin, all at 1.0 mg/mL. After 8 weeks, there were no significant differences in plasma triglyceride levels for the mice in the different treatment groups (water, 142±16 mg/dL; egg lecithin, 138±18 g/dL; soy lecithin, 161.6±8.5 mg/dL; and DMPC, 158.4±6.3 mg/dL). Total cholesterol was also not different (water, 569±97 mg/dL; egg lecithin, 549±132 mg/dL; soy lecithin, 576±86 mg/dL; and DMPC, 587±112 mg/dL). Total plasma phospholipid levels were not significantly different among the groups (water group, 318±28 mg/dL; soy lecithin, 332±17 mg/dL; egg lecithin, 327±23 mg/dL; and DMPC, 341±27 mg/dL). HDL phospholipid levels were 85.2±7.3, 88.1±2.9, 84.2±2.9, and 200.5±11.3 mg/dL in the groups on water, egg lecithin, soy lecithin, and DMPC, respectively, indicating a 2.3-fold increase ($P=0.003$) in plasma HDL phospholipids in the mice receiving DMPC. DMPC was not found in the plasma of any mice except those given DMPC. Plasma DMPC concentration in the group that received DMPC in their drinking water was 84±74 μg/dL, or 0.02% of the total plasma phospholipid concentration. Plasma free fatty acid concentrations after an overnight fast were not significantly different between the groups. The values were 41.5±4 mg/dL for mice receiving

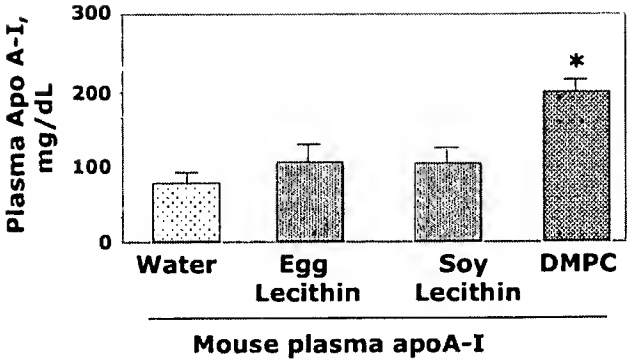


Figure 1. DMPC increases plasma apoA-I levels. Four-week-old female apoE-null mice received drinking water alone (Water) (n=12), egg lecithin (n=8), soy lecithin (n=8), or DMPC (n=8), all at 1.0 mg/mL. After 8 weeks, mice were bled, and plasma was used to determine apoA-I levels. Plasma apoA-I levels were determined by a specific ELISA. *$P<0.05$, statistically significant difference.

water alone, 36.3±5 mg/dL for mice receiving soy lecithin, 37.0±3 mg/dL for mice receiving egg lecithin, and 38.9±2 mg/dL for mice receiving DMPC. HDL cholesterol levels for the mice receiving soy lecithin or egg lecithin were not significantly different from those for the mice receiving water alone, but there was a 2.3-fold ($P=0.003$) increase in HDL cholesterol levels in the mice that received DMPC in their drinking water (water, 14.1±3.2 mg/dL; egg lecithin, 16.2±4.3 mg/dL; soy lecithin, 18.1±4.4 mg/dL; and DMPC, 32.4±5.1 mg/dL). There was a corresponding increase in plasma apoA-I levels in the mice that received DMPC as determined by ELISA (Figure 1), which was confirmed by Western blot analysis (data not shown). Thus, the major effect of DMPC appears to be on the induction of apoA-I, paraoxonase activity, and HDL cholesterol and phospholipid levels in the plasma. There were no significant differences in body weight or in the wet weight of liver, heart, and kidneys in the animals receiving the different treatments (data not shown).

As shown in Figure 2, when DMPC or soy or egg-yolk lecithin at 1.0 mg/mL was added to the drinking water of 4-week-old apoE-null mice for 8 weeks, HDL function was dramatically improved in the mice receiving DMPC but not in those receiving lecithin.

Figure 3 demonstrates that there was a significant reduction in aortic sinus lesions in the mice receiving DMPC but not in the mice receiving either egg-yolk or soy lecithin.

In other experiments, 1 group of 10-month-old female apoE-null mice maintained on a chow diet were killed and found to have lesion scores of 405 198±77 231 μm^2 (Figure 4A). A second group of these mice was continued on the chow diet for an additional 5 weeks, with no additions to their drinking water. After the additional 5 weeks, the lesion scores of this second group were 471 233±64 521 μm^2 (Figure 4A). A third group of the 10-month-old mice was continued on the chow diet for another 5 weeks, but DMPC was added to their drinking water at 1.0 mg/mL. After the additional 5 weeks, the lesion scores of this third group that had received DMPC were 267 042±42 105 μm^2 ($P=0.044$ compared with the first

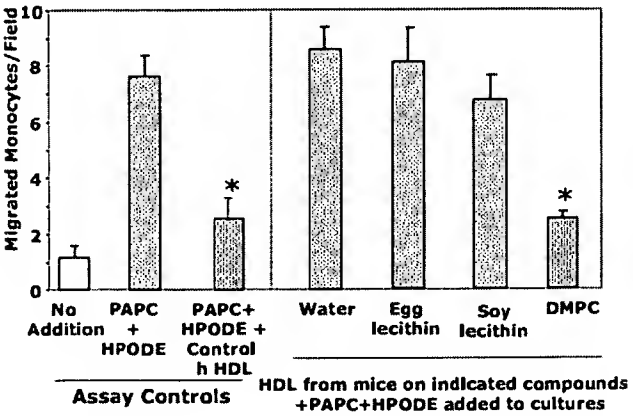


Figure 2. DMPC improves HDL function. Four-week-old female apoE-null mice received drinking water alone (Water) (n=12), egg lecithin (n=8), soy lecithin (n=8), or DMPC (n=8), all at 1.0 mg/mL. After 8 weeks, mice were bled, and HDL was isolated by FPLC. PAPC 20 μg and HPODE 1.0 μg were added or not added (No Addition) to human artery wall cell cocultures with normal human HDL at 350 μg cholesterol (Control h HDL) or murine HDL at 50 μg cholesterol from mice that had received drinking water without additions (Water) or drinking water with egg lecithin, soy lecithin, or DMPC at 1.0 mg/mL. After 8 hours, supernatants were collected and assayed for monocyte chemotactic activity. Data are mean±SD of number of migrated monocytes in 9 fields for triplicate samples from each of 2 separate experiments. *$P<0.05$, statistically significant difference.

group and $P=0.003$ compared with the second group) (Figure 4A).

To study the action of the phospholipids in male mice, groups of 6-month-old male apoE-null mice (n=8 per group) were provided with water alone, egg lecithin, soy lecithin, or DMPC, all added to the drinking water at 1.0 mg/mL. After 8 weeks on this treatment, the lesion scores for the aortic root serial sections were not different for mice given water, egg lecithin, or soy lecithin (Figure 4B). However, the mice given DMPC had significantly smaller lesion scores (Figure 4B; $P=0.0031$ for the DMPC group compared with the water control group). A representative aortic sinus section is shown for the water control group (Figure 4C) and for mice receiving DMPC (Figure 4D). Aortic sections from the mice that received egg lecithin or soy lecithin were similar to the

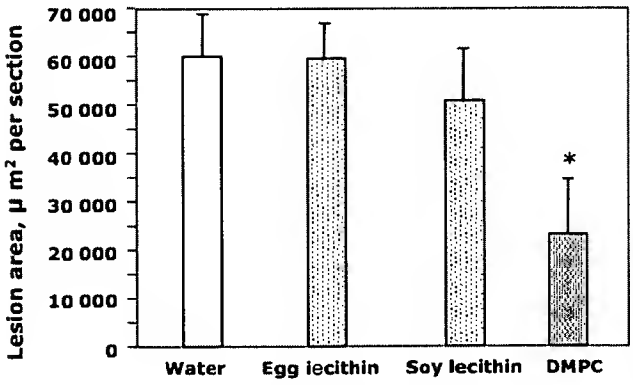


Figure 3. DMPC prevents lesion formation. Mice described in Figures 1 and 2 were killed after bleeding, and their aortic sinus lesion scores were determined as in Methods. Data are mean±SD. *$P=0.021$.

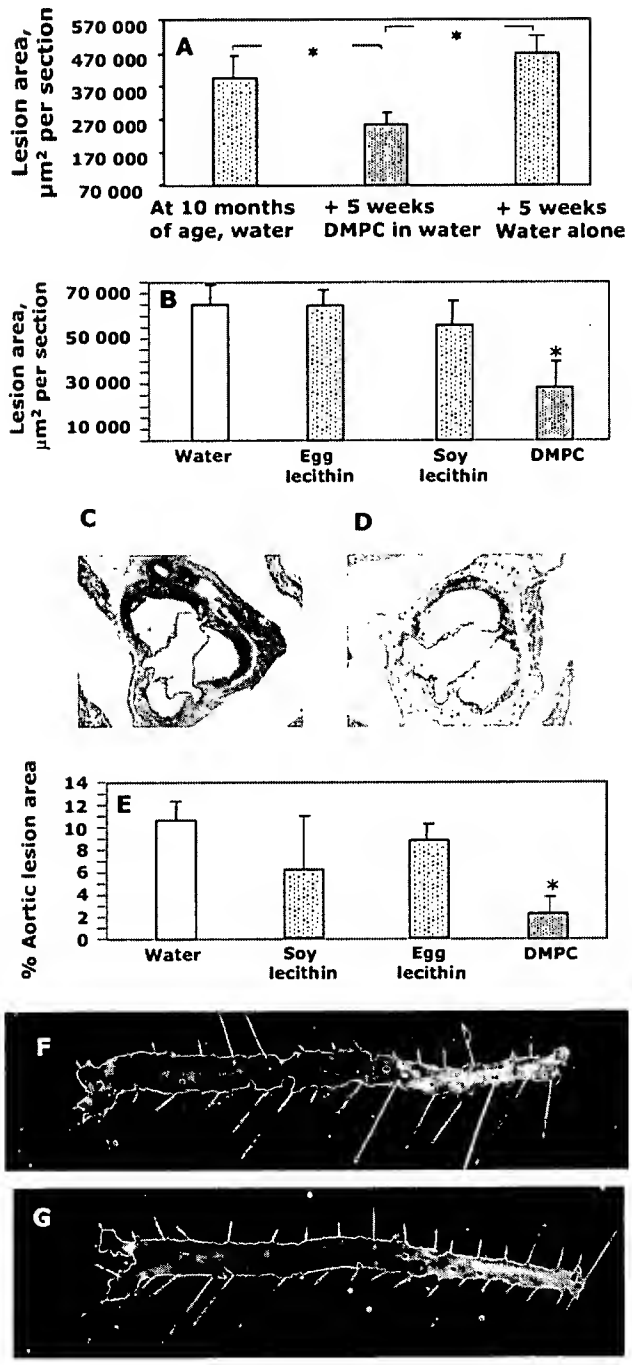


Figure 4. DMPC causes lesion regression. A, Nineteen female apoE-null mice were maintained on a chow diet for 10 months. After 10 months, 1 group of 5 mice were killed and aortic sinus lesion scores determined as in Methods (At 10 months of age, water). A second group of 8 mice were continued on chow diet, but DMPC at 1.0 mg/mL was added to their drinking water for an additional 5 weeks, at which time mice were killed and aortic lesion scores determined (+5 weeks DMPC in water). A third group of 6 mice was continued on chow diet with no additions to their drinking water for 5 weeks longer, at which time mice were killed and aortic lesion scores determined (+5 weeks Water alone). *P=0.044 for +5 weeks DMPC in water vs first group (At 10 months of age, water) and *P=0.003 vs third group (+5 weeks water alone). B, Groups of 6-month-old male apo E-null mice on laboratory chow were provided with water alone, egg lecithin, soy lecithin, or DMPC, all at 1.0 mg/mL in their drinking water for 8 weeks. Lesion scores were then determined for serial sections of aortic root. *P=0.0031, statistically significant difference for DMPC group vs group maintained on water alone. C and D, Representative aortic sinus section for water control group (C) and for mice receiving DMPC (D). E, Entire aortas of mice described in B were removed, fixed, and stained, and en face lesion scores were determined as described in Methods. *P=0.001, statistically significant difference between values for DMPC-treated mice vs water alone. F and G, Representative aorta en face for water control group (F) and for mice receiving DMPC (G).

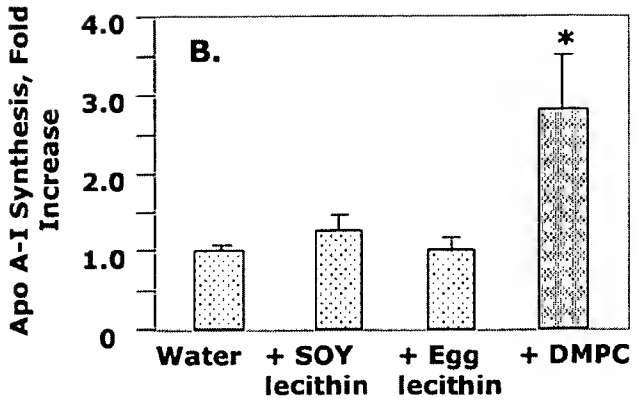


Figure 5. DMPC increases intestinal apoA-I synthesis. Groups of 4-month-old male apoE-null mice (n=4 per group) were provided with water, soy lecithin, egg lecithin, or DMPC, all at 1.0 mg/mL in their drinking water. After 18 hours, mice were killed and apoA-I synthesis was determined in jejunum mucosa as described in Methods. *P=0.011.

water control group (data not shown). The correlation coefficient for aortic sinus lesions scores versus plasma HDL cholesterol levels was −0.894, and the correlation coefficient for aortic sinus lesion scores and plasma apoA-I levels was −0.945. In addition, en face lesion area for the entire aortic surface was determined in these male mice and was found to be significantly less only in the mice that received DMPC (Figure 4E; P=0.001 for the DMPC group compared with the water control group). A representative aorta en face is shown for the water control group (Figure 4F) and for mice receiving DMPC (Figure 4G). Aortas from the mice that received egg lecithin or soy lecithin were similar to the water control group (data not shown). The correlation coefficients for en face lesion area for the entire aortic surface versus plasma HDL cholesterol levels or plasma apoA-I levels were −0.991 and −0.967, respectively.

As shown in Figure 5, adding DMPC at 1.0 mg/mL to the drinking water of apoE-null mice significantly increased apoA-I synthesis in the jejunum of male mice compared with water alone, and adding soy or egg lecithin to the drinking water at the same concentration did not significantly alter jejunal apoA-I synthesis. Similar results were found in female mice (data not shown).

Discussion

When the apoE-null mice were allowed access to DMPC for 24 hours, particles containing cholesterol and paraoxonase 1 activity were found in the region to the right of HDL in the FPLC chromatogram, suggesting that DMPC induced the formation of pre-β-HDL–like particles, which in the absence

of continued access to DMPC appeared to move into the mature HDL fractions over the next 24 hours.

The increase in paraoxonase activity together with increased intestinal synthesis of apoA-I (Figure 5) and the increase in plasma apoA-I (Figure I) may have contributed to the decreased atherosclerosis (Figures 3 and 4) seen in the mice receiving DMPC. The very strong negative correlation coefficients between lesion area and plasma HDL cholesterol levels and plasma apoA-I levels indicate an inverse correlation between plasma HDL cholesterol and apoA-I levels and lesions in this model of mouse atherosclerosis and are consistent with this hypothesis. We previously reported that infusion of apoA-I into both mice and humans decreased LDL oxidation in our human artery wall coculture model.[20,21] We also recently reported that an apoA-I mimetic peptide synthesized from all D-amino acids when given orally improved HDL function and dramatically reduced atherosclerosis in both LDL receptor–null and apoE-null mice.[16]

DMPC prevented lesion formation (Figure 3) and appeared to cause lesion regression (Figure 4). The increase in apo A-I synthesis after DMPC (Figure 5) is the presumed reason for the marked increases seen in HDL cholesterol levels. This and the dramatic improvement in the ability of HDL to prevent the induction of monocyte chemotactic activity in response to oxidized phospholipids in the artery wall model system (Figure 2) are probably the main causes of the marked reduction in lesions, because total plasma cholesterol, triglycerides, phospholipids, and fatty acids were not significantly altered by any of the treatments. In addition, the antiatherogenic response to DMPC would not be predicted from the known effects of myristic acid, which has proatherogenic properties.[24] Because the mice drank ≈2.5 mL/d per mouse, we can assume that the mice received ≈2.5 mg of DMPC per mouse in the experiments described in Figures 1 to 4. The equivalent dose for a 70-kg human would be 8.75 g/d of DMPC. The usual dose of lecithin recommended as a food supplement is on the order of 10 g/d. Most humans seem to tolerate up to 25 g/d of lecithin without serious side effects.[12]

The molecular mechanisms by which DMPC (but not soy or egg lecithin) increases HDL cholesterol, improves HDL function, increases apoA-I synthesis in the intestine, raises plasma apoA-I, and prevents atherosclerosis or causes its regression in apoE-null mice remain to be elucidated. This report, together with our previous report,[16] suggests that oral HDL–based therapies may hold considerable therapeutic promise.

Acknowledgments

This work was supported in part by US Public Health Service grant HL-30568 and the Laubisch, Castera, and M.K. Gray Funds at the University of California Los Angeles.

References

1. Huang W, Saski J, Matsunaga A, et al. A single amino acid deletion in the carboxy terminal of apolipoprotein A-I impairs lipid binding and cellular interaction. *Arterioscler Thromb Vasc Biol.* 2000;20:210–216.
2. O'Brien BC, Corrigan SM. Influence of dietary soybean and egg lecithins on lipid responses in cholesterol-fed guinea pigs. *Lipids.* 1988;23:1–14.
3. Wilson TA, Meservey CM, Nicolosi RJ. Soy lecithin reduces plasma lipoprotein cholesterol and early atherogenesis in hypercholesterolemic monkeys and hamsters: beyond linoleate. *Atherosclerosis.* 1998;140: 147–153.
4. Jimenez MA, Scarino ML, Vignolini F, et al. Evidence that polyunsaturated lecithin induces a reduction in plasma cholesterol level and favorable changes in lipoprotein composition in hypercholesterolemic rats. *J Nutr.* 1990;120:659–667.
5. Navder KP, Baraona E, Leo MA, et al. Oxidation of LDL in baboons is increased by alcohol and attenuated by polyenylphosphatidylcholine. *J Lipid Res.* 1999;40:983–987.
6. Greten H, Raetzer H, Stiehl A, et al. The effect of polyunsaturated phosphatidylcholine on plasma lipids and fecal sterol excretion. *Atherosclerosis.* 1980;36:81–88.
7. Childs MT, Bowlin JA, Ogilvie JT, et al. The contrasting effects of a dietary soya lecithin product and corn oil on lipoprotein lipids in normolipidemic and familial hypercholesterolemic subjects. *Atherosclerosis.* 1981;38:217–228.
8. Vroulis G, Smith RC, Schoolar JC, et al. Reduction of cholesterol risk factors by lecithin in patients with Alzheimer's disease. *Am J Psychiatry.* 1982;139:1633–1634.
9. Brook JG, Linn S, Aviram M. Dietary soya lecithin decreases plasma triglyceride levels and inhibits collagen- and ADP-induced platelet aggregation. *Biochem Med Metab Biol.* 1986;35:31–39.
10. Kesaniemi YA, Grundy SM. Effects of dietary polyenylphosphatidylcholine on metabolism of cholesterol and triglycerides in hypertriglyceridemic patients. *Am J Clin Nutr.* 1986;43:98–107.
11. O'Brien BC, Andrews VG. Influence of dietary egg and soybean phospholipids and triacylglycerols on human serum lipoproteins. *Lipids.* 1993; 28:7–12.
12. Wood JL, Allison RG. Effects of consumption of choline and lecithin on neurological and cardiovascular systems. *Fed Proc.* 1982;41:3015–3021.
13. Knuiman JT, Beynen AC, Katan MB. Lecithin intake and serum cholesterol. *Am J Clin Nutr.* 1989;49:266–268.
14. Wang H, Roberson R, Du J, et al. Regulation of apolipoprotein secretion by biliary lipids in newborn swine intestinal epithelial cells. *Am J Physiol.* 1999;276:G353–G362.
15. Wang H, Du J, Lu S, et al. Regulation of intestinal apolipoprotein A-I synthesis by dietary phosphatidylcholine in newborn swine. *Lipids.* 2001; 36:683–687.
16. Navab M, Anantharamaiah GM, Hama S, et al. Oral administration of an apo A-I mimetic peptide synthesized from D-amino acids dramatically reduces atherosclerosis in mice independent of plasma cholesterol. *Circulation.* 2002;105:290–292.
17. Navab M, Hama SY, Hough GP, et al. A cell-free assay for detecting HDL that is dysfunctional in preventing the formation of or inactivating oxidized phospholipids. *J Lipid Res.* 2001;42:1308–1317.
18. Tangirala RK, Rubin E, Palinski W. Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. *J Lipid Res.* 1995; 36:2320–2328.
19. Tangirala RK, Tsukamoto K, Chun S, et al. Regression of atherosclerosis induced by liver-directed gene transfer of apolipoprotein A-I in mice. *Circulation.* 1999;26:1816–1822.
20. Navab M, Hama SY, Cooke CJ, et al. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: step 1. *J Lipid Res.* 2000;41:1481–1494.
21. Navab M, Hama SY, Anantharamaiah GM, et al. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: steps 2 and 3. *J Lipid Res.* 2000;41:1495–1508.
22. Van Lenten BJ, Wagner AC, Anantharamaiah GM, et al. Influenza infection promotes macrophage traffic into arteries of mice that is prevented by D-4F, an apolipoprotein A-I mimetic peptide. *Circulation.* 2002;106:1127–1132.
23. Escola-Gil JC, Julve J, Marzal-Casacuberta A, et al. Expression of human apolipoprotein A-II in apolipoprotein E-deficient mice induces features of familial combined hyperlipidemia. *J Lipid Res.* 2000;41:1328–1338.
24. Kummrow E, Hussain MM, Pan M, et al. Myristic acid increases dense lipoprotein secretion by inhibiting apoB degradation and triglyceride recruitment. *J Lipid Res.* 2002;43:2155–2163.